IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Edwards et al.		
Appln. No.	:	09 680,738	Examiner:	B. Loeb
Filed	:	October 6, 2000	Group:	1636
For	:	ADJUSTABLE SENSITIVITY GENETIC MOLECULAR INTERACTION SYSTEMS, INCLUDING PROTEIN-PROTEIN INTERACTION SYSTEMS FOR DETECTION AND ANALYSIS		

DECLARATION UNDER 37 C.F.R. 1.132

Considered
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I hereby certify that this paper is being deposited with the United States Postal Service as Express Mail in an envelope bearing label number EL923191342US and addressed to: Commissioner for Patents, Arlington, VA 22202, on

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Assistant Commissioner for Patents
Washington, D.C. 20231

Sir,

I , David Edwards residing at 3800 Spring Valley Road, # 804 Addison, Texas, declare as follows:

1. I am a co-inventor of the above-identified patent application.

2. Subsequent to the filing, I have prepared and circulated for investment and marketing purposes the attached explanation of the kinetics of the "Adjustable Sensitivity Genetic Molecular Interaction System" of the above-identified application.

3. The entire subject matter of the attached explanation merely describes the inherent properties of the invention disclosed in the above-identified application and adds no new subject matter to such application.

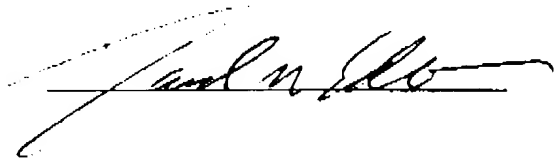
4. I declare further that all statements made herein of may own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant application.

_____

David Edwards

Date: _____

4. I declare further that all statements made herein of may own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant application.



David Edwards

Date: December 10, 2001

Hybrigen

HybrEXCEL™ Interaction Hybrid Systems- Understanding interaction as a kinetic equilibrium.

HybrEXCEL, which is covered by US and international patent filings, is a proprietary technology that transforms two-hybrid and other *in vivo* interaction detection technologies into sensitive and reliable detectors of protein binding partners. It is a powerful overlay for any genetic interaction detection system.

The principle behind essentially all genetic interaction detection systems is that two test proteins can unite two separated modular domains to restore function to a biologically active molecule. In the original nuclear yeast systems, this was a transcription factor; many variations have now been designed. Output is measured by an *in vivo* reporter, e.g. an enzyme that, when transcribed, allows growth of a mutant yeast strain auxotrophic for a simple metabolite.

Using two-hybrid technology, researchers have found a number of novel and verifiable interactions and have established the tremendous potential of genetic interaction systems. All too often, however, users of these systems have faced the frustration of finding many non-specific interactors for every meaningful interactor, necessitating extensive secondary screening on a case-by-case basis. Furthermore, in many cases such screens have failed to detect any specific interactions. These deficiencies make the generally available two-hybrid systems poor choices for large-scale application, as was readily apparent in recent genome-wide pilot studies by Curagen and RIKEN (Ito *et al.*, April 10, 2001, *PNAS*, vol. 98.8, 4569-4574.)

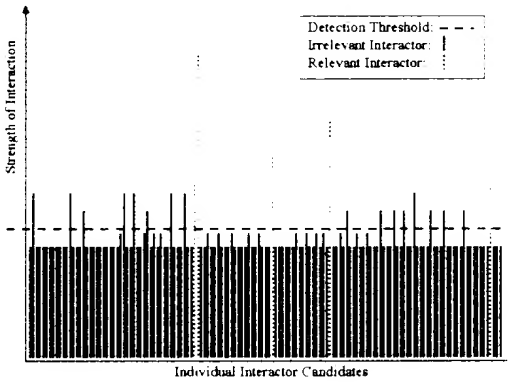


Figure 1: Schematic of Interaction Set plotted as strength of interaction with a given "perfect" bait.

The limitations of the first generation two-hybrid systems reflects the underlying assumption that there either is or is not an interaction. In truth, protein-protein interactions are best described as equilibria, with specific interactions differing from non-specific ones in the amount of time the two proteins spend in close proximity to one another. Although the range of this variation will vary for different test proteins, available genetic interaction detection systems have been designed with a preset level of sensitivity. They will thus miss significant interactions in some cases, while detecting many insignificant interactions in others.

Empirically, one finds that on average only one in ten test proteins fits the profile shown in Figure 1, in which the preset threshold of detection lies well above the majority of the irrelevant and non-specific interactions, but below that of the sought-after specific interactions. In the sections that follow, we illustrate the difficulties encountered in the vast majority of two-hybrid screens and explain how the HybrEXCEL system overcomes these problems.

Technical Limitation One: False Positives.

The most widely acknowledged limitation of the two-hybrid system is the large number of false positives that result from most screens.

There are many current and generally unsatisfactory procedures for avoiding this pitfall. There are false positive databases, statistical models, computer models, reversing the orientation of the interactors, etc. There are leaking systems that require poisoning the reporter to prevent interactions from being detected. There is even the titration of nutrients to try to limit or encourage the leak-through of non-specific interactions. None of these approaches takes advantage of the basic understanding of a kinetic equilibrium to encourage specific interactions over competing non-specific proteins.

Figure 2 depicts the same interaction pattern as Figure 1, except that essentially all of the interactions fall at the preset detection threshold of the standard two-hybrid system. The HybrEXCEL technology does not change the strength of the interactions, but instead renders the threshold of detection adjustable for that interaction system. Without modifying the actual reporters, the HybrEXCEL technology can encourage all interactions in parallel to be more or less likely to be detectable above the threshold. Effectively, the threshold of detection can be moved up or down, as shown in Figure 3. In practice, Hybrigen raises the threshold until it is possible to select a level of detection that best demonstrates a favorable signal-to-noise ratio before analyzing any of the individual clones.

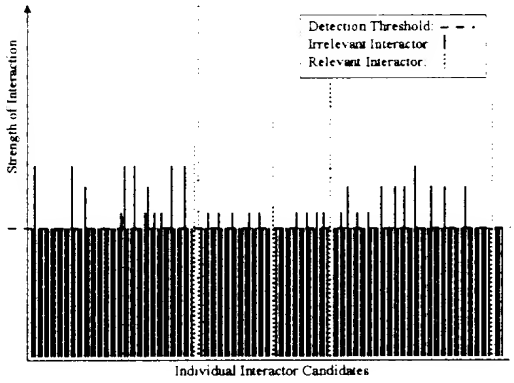


Figure 2: Schematic of the Interaction Set plotted versus strength of interaction for a hypothetical promiscuous bait used in an unmodified two-hybrid system.

Most biologically relevant interactors interact more specifically than the majority of non-biologically relevant interactors. This is the reason two-hybrid and other interaction detection systems work at all. However, certain protein baits tend to interact non-specifically more strongly with numerous preys, giving false positives.

Choosing positives from an interaction screen is a matter of recognizing the probabilities; the more exclusive a detectable event is in the screen, the more likely the event is real and specific. A random screen, and indeed even the cell itself, contains millions of non-interacting proteins that serve as negative controls. Detection of an interaction is not binary, but is rather a dynamic event in which all the proteins in the cell compete with the

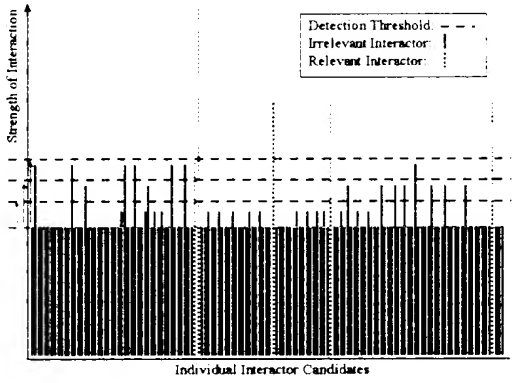


Figure 3: Schematic of the Interaction Set plotted versus strength of interaction for a hypothetical promiscuous bait and the threshold raised for decreased sensitivity.

prey for the interaction sites on the bait. These are the primary negative controls of any interaction system. If the bait-prey interaction is more specific than for the random proteins in the cellular milieu, it may be strong enough to activate the reporter. However, if ten percent of the preys in a library were to give positives, there would clearly be a difficulty in analyzing the data. It would be highly unlikely for a single bait relevantly to interact with 100,000 individual prey proteins, yet this can occur for certain baits in a non-adjustable detection system.

As a primary step of eliminating false positive interactions, Hybrigen takes advantage of the equilibrium of the protein-protein interaction to encourage or discourage reporting of a given interaction pair, relative to all other non-specific interactions. That is, we change the threshold for which all interactions are reported across all interactions tested. Therefore, before ranking an interaction as a preliminary positive, it is directly compared to the specificity of over 10^7 other pairs simultaneously.

The reporters in most genetic interaction detection systems are fixed and limiting; for example, there is often far more DNA-binding peptide expressed than reporter sites available to bind. In order to encourage specificity, HybrEXCEL allows controlled overexpression of the bait versus the prey. For interactions with a very high affinity (low K_d), the likelihood that the bait will occupy the reporter site at a given moment and will also be bound to a prey molecule remains high. At very low K_d, essentially every bait is bound to a prey molecule at a given moment, and it is difficult to discourage reporting of this very strong interaction.

The lower the affinity (higher the K_d) the less likely the interaction will be detected under those same circumstances. For example, if there is a one-to-one ratio of bait to prey but the K_d is high, few of the bait molecules will be bound at a given moment, so the likelihood of activity at the reporter decreases. Thus, sensitivity can be decreased until only very specific interactions remain detectable.

Interestingly, there does remain a subset of non-biologically relevant interactors that come through even adjustable selections and screens. For example, Hybrigen often detects Cytochrome C fragments across multiple species and pathways. Hybrigen uses such interactions, often the subject of the negative control databases, as controls.

Routinely, Hybrigen performs assays at maximum threshold in which every molecule detected is a biologically relevant positive. In these cases, the ideal threshold is lower so that moderately robust interactions are not neglected. In the most dramatic example to date, the range of maximum to minimum sensitivity gave a 1,000 fold decrease in detected signal; for a mutated form the mouse protein RhoA, two percent (approximately 200,000) of all the assayed pairs fell above threshold at maximum sensitivity while only 200 positives were detected at minimum sensitivity. Analysis revealed that the subset of known RhoA interactors present in the library were successfully detected and recovered at minimum sensitivity.

Technical Limitation Two: False Negatives.

The least often considered but possibly most important limitation of the two-hybrid and other interaction systems is the potential to miss relevant interactions.

Previously, there were no satisfactory methods for detecting interactions that fall below the auxotrophic threshold of even the most sensitive two-hybrid systems. Figure 4 depicts the same interaction pattern as in Figure 1, except that all but one of the interactions fall below the preset detection threshold of the standard two-hybrid system. In some cases, all of the interactions fall below this threshold, as is often the case when screening discrete protein subdomains, especially the intracellular domains of transmembrane receptors.

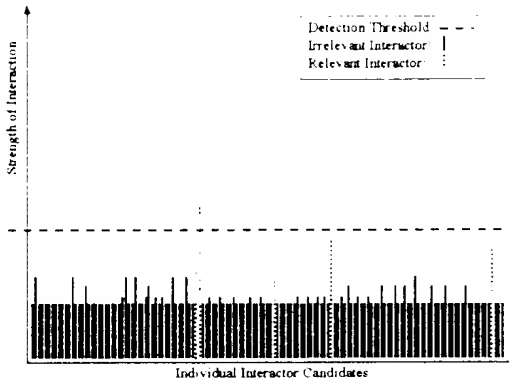


Figure 4: Schematic of the Interaction Set plotted versus strength of interaction for a hypothetical weakly interacting bait in an unmodified two-hybrid system.

As an overlay for a given interaction system, the HybrEXCEL technology does not change the strength of the interactions, but instead renders the threshold of detection adjustable for that interaction system. Without modifying the actual reporters, the HybrEXCEL technology can encourage all interactions in parallel to be more or less likely to be detectable above the threshold. Effectively the threshold of detection can be moved up or down, as shown in Figure 5. Hybrigen uses HybrEXCEL to lower the threshold until noise is detected and then selects the level of detection that best demonstrates a favorable signal-to-noise ratio before analyzing any of the individual clones.

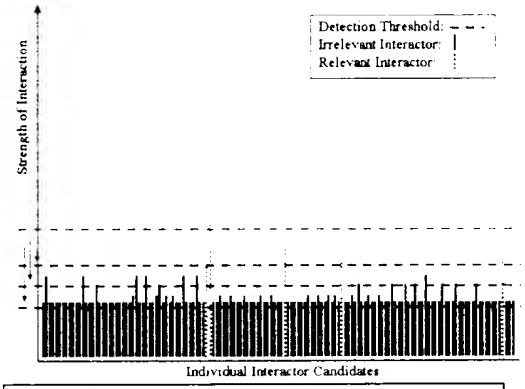


Figure 5: Schematic of the Interaction Set plotted versus strength of interaction for a hypothetical weakly interacting bait and the threshold lowered for more sensitivity.

Again, HybrEXCEL takes advantage of the equilibrium of the protein-protein interaction to encourage or discourage reporting of a given interaction pair, relative to all other non-specific interactions. Hybrigen changes the threshold for which all interactions are reported across all interactions tested and directly compares them to the specificity of the other 10^7 pairs simultaneously.

In order to encourage detection of an interaction, HybrEXCEL allows controlled overexpression of the prey versus the bait. For interactions with a very low affinity (high K_d), the likelihood that the bait will occupy the reporter site at a given moment and will also be bound to a prey molecule increases as the concentration of the prey increases. Across all 10^7 pairs assayed in parallel, the most specific interactions cross the threshold for detection first.

The intracellular domains of transmembrane receptors provide good examples of baits that often interact only weakly with biologically relevant molecules but that can be successfully used in a HybrEXCEL Interaction System. Hybrigen has a rapidly growing list of protein interactions and interactors in well-studied pathways that were missed precisely because investigators used fixed

threshold systems. It is established opinion in molecular biology that often the weak interactions (highest K_d) are the most interesting.

Q&A:

Does Hybrigen still find false positives and miss real positives?

Since interaction detection is a function of probability, even HybrEXCEL will generate some false positives. However, HybrEXCEL yields a much better assessment of the probabilities with minimal analysis. We eliminate most remaining false positives using our unique false positive database. When used in conjunction with Hybrigen's TriACT™ Library System (described in a separate document), we find that the existing false positive databases are lacking many of the promiscuous domains, since those domains have never been assayed using current library technology.

If there are interactions that absolutely require several components to interact together, HybrEXCEL will not always detect the interaction. However, our very fine control of sensitivity has allowed us to detect weak interactions that usually require other components, that are transient, or that require covalent modifications in order to be detected in an unmodified interaction system. Many interactions do not disappear when assayed out of context, they simply weaken.

What else can Hybrigen not find?

Interaction detection systems do not detect one-time interactions, such as proteolytic events. We employ alternative technologies to move past these events in a pathway cascade.

What about extracellular interactions?

Extracellular interactions generally do not work well in any interaction system. They can be found, but glycosylation and other modifications common to extracellular proteins and the varying milieu of the extracellular compartment is not duplicated by the intracellular genetic detection systems we modify with HybrEXCEL.

What methods of adjustability and filtering does Hybrigen employ?

Our proprietary system for adjusting the detection threshold for interaction detection is described above. We also employ a proprietary false positive database, proprietary algorithms, and basic probability models to identify and rank the interactors we find.

Does Hybrigen screen full-length proteins, protein domains, or peptides when looking for interactors?

Yes. All of the above can and are screened with HybrEXCEL technology.

In which interaction systems does Hybrigen prefer to employ its HybrEXCEL technology in its analysis of protein interactions?

We have found that the various yeast-based interaction systems are ideal for most protocols, with a nuclear transcription-based system lending itself best to maximum sensitivity. Bacterial systems appear to give up the appropriate eukaryotic milieu. Mammalian systems remain unwieldy outside specific binary assays.

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